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Preclinical Applications of Multi-Platform Imaging in Animal Models of Cancer

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Abstract

In animal models of cancer, oncologic imaging has evolved from a simple assessment of tumor location and size to sophisticated multi-modality exploration of molecular, physiological, genetic, immunological and biochemical events at microscopic to macroscopic levels, performed non-invasively and sometimes in real time. We briefly review animal imaging technology and molecular imaging probes together with selected applications from recent literature. Fast and sensitive optical imaging is primarily used to track luciferase-expressing tumor cells, image molecular targets with fluorescent probes, and report on metabolic and physiological phenotypes using smart switchable luminescent probes. MicroPET/ SPECT have proven to be two of the most

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translational modalities for molecular and metabolic imaging of cancers: Immuno-PET is a promising and rapidly evolving area of imaging research. Sophisticated MRI techniques provide high-resolution images of small metastases, tumor inflammation, perfusion, oxygenation and acidity. Disseminated tumors to the bone and lung are easily detected by microCT, while ultrasound provides real-time visualization of tumor vasculature and perfusion. Recently available photoacoustic imaging provides real time evaluation of vascular patency, oxygenation, and nanoparticle distributions. New hybrid instruments such as PET-MRI promise more convenient combination of the capabilities of each modality, enabling enhanced research efficacy and throughput.

Recent technological developments in scanner design and advances in image reconstruction have secured the rapid application of noninvasive imaging for detection, characterization and monitoring of cancer etiology in a variety of animal models (1–3). Obvious advantages arise from the ability to study structure, metabolism and function of cancer cells and cancer supporting microenvironment longitudinally, without the need for necropsy. Indeed, imaging is non-invasive and repetitive studies are performed in the same animals, with each animal serving as its own control. Importantly, most imaging platforms can efficiently survey whole animals, opening new horizons for studying metastatic disease. Furthermore, many imaging technologies are intrinsically translational by applying identical imaging protocols, imaging tracers and image analysis to various species, thereby providing a bridge from laboratory animals to companion animals and ultimately to humans with the goal of easing the burden of human cancer (4–6). There are various imaging platforms, also referred to as imaging modalities, each based on a specific physical principle (Table 1A), allowing unique information/data to be generated. The primary reason for applying a multi-platform imaging approach to cancer research is to obtain comprehensive information from a cancer-bearing animal (Table 1B). The in-vivo cancer imaging modalities are highly complementary, providing a variety of quantitative biomarkers for cancer cell tracking, and assessing tumor dimensions, pathophysiology, metabolism and molecular composition (Table 1B, Figure 1), but each has specific advantages and weaknesses (6–8). In this review, we highlight the state-of-the-art applications of pre-clinical multi-modal multi-scale imaging and focus on the specific applicability to cancer research.

Magnetic Resonance Imaging and Spectroscopy (MRI/MRS):

MR physics is complicated, but offers extraordinary opportunities to manipulate tissue water signals based on relaxation mechanisms, chemical exchange, flow and diffusion to reveal diverse anatomical, physiological and cellular properties of cancer at high external magnetic fields. The most sensitive nucleus is the proton, notably in H_2O.

Anatomical MRI:

Among all imaging modalities, MRI possesses the best soft tissue contrast, which may be enhanced still further using exogenous paramagnetic contrast agents. Excellent spatial resolution can reveal ultra-small cancer lesions (as small as 0.2 mm diameter with 9.4 T MRI), particularly in well-structured tissues such as the brain. MRI is the “gold-standard” for orthotopic brain tumors and brain metastases (Figure 1–A1, (9–13)), and is also widely

applied for the detection of other soft tissue lesions including liver (Figure 1–A2) and lung metastases (Figure 1–A3).

Physiological MRI:

Beyond high-resolution anatomical MRI, tumor cellular density and edema are easily quantified using diffusion-weighted MRI, which is sensitive to restricted or enhanced diffusion of water molecules, respectively (Figure 1–B7) (2, 14, 15). Several recent publications reported increased apparent diffusion coefficients (ADCs) associated with treatment-induced necrosis (16–19). Tissue oxygenation may be examined using oxygen–sensitive MRI. Notably, apparent transverse relaxation rate (R_2*) is sensitive to the concentration of deoxyhemoglobin, as exploited in blood oxygen level dependent (BOLD) contrast and forming the basis of fMRI to assess neurological activation (20). Meanwhile, so-called tissue oxygen level dependent (TOLD) MRI exploits the sensitivity of the spin-lattice relaxation rate, R_1, to the paramagnetic oxygen molecule (O_2) itself (Figure 1–B8) (21–27). Noting the importance of hypoxia in cancer development, aggressiveness and response to therapy, an oxygen gas breathing challenge has been shown to provide a simple effective theranostic: well oxygenated tissues show response to an oxygen-gas breathing challenge, whereas hypoxic tissue does not (28). This approach has been demonstrated to provide a prognostic imaging biomarker in rats with respect to stereotactic ablative radiation therapy (SABR) (24, 28) and is feasible in man (21, 29).

Vascular MRI:

The use of exogenous MR contrast agents, namely gadolinium chelates as T_1- and iron oxide nanoparticles as T_2-contrast, enables imaging of tumor angiogenesis and changes in tumor vascularity. Intravenous injection of gadolinium contrast agent allows direct visualization of tumor vasculature by magnetic resonance angiography (MRA, Figure 1–C11) (30) or the generation of tumor perfusion/ permeability K^{trans} maps using dynamic-contrast enhanced (DCE)-MRI (Figure 1–C12) (31–33). The use of T_2-contrast blood pool agents (based on ferumoxytol and other iron oxide nanoparticles) allows susceptibility-contrast imaging to assess tumor blood volume (32, 34).

Cellular and Receptor MRI:

The same iron oxide nanoparticles can be used for cell tracking. Breast cancer cells prelabelled with ferumoxytol in-vitro, could be detected in the brain by T_2-MRI following IV injection (Figure 1–E21) (35). Meanwhile, injection of ferumoxytol itself leads to extensive uptake by macrophages, which has been observed as reduced T_2-signal, revealing M1 (anti-tumor) or M2 (pre-tumor) activity (Figure 1–E22) (36–38). Some reports have explored the possibilities of using iron oxide- or gadolinium-based contrast for detecting cell receptors including HER2 or C2 imaging in mouse models of breast cancer and pre-cancerous renal inflammation (39–41). In mouse prostate cancer models, PSMA-receptors have been successfully imaged using targeted iron oxide nanoparticles by T_2-MRI or a diamagnetic dextran-based CEST MRI agent (see below) (42–44). Receptor imaging with MRI poses unique challenges for signal amplification to deposit sufficient MRI contrast per receptor molecule for its detection.

Other nuclei and metabolic MRS:

Beyond proton MRI of tissue water, spectroscopic imaging can detect several endogenous metabolites that occur at sufficiently high concentrations, such as lactate, glutamine, glutamate, creatine, N-acetyl aspartate (NAA), gamma-aminobutyric acid (GABA), citrate, choline, and, most recently, 2-hydroxyglutarate (2HG) (45). The oncometabolite 2-HG accumulates in low-grade glioma, secondary glioblastoma, and acute myeloid leukemia, owing to mutations in the metabolic enzymes isocitrate dehydrogenase (IDH) 1 or 2. Mutant IDH1/2 aberrantly produces 2-HG (instead of ketoglutarate), which is detectable by ^{1}H-MRS or ^{13}C-MRSI following hyperpolarized [1-^{13}C]-glutamine administration (Figure 1–D17) (46). For ^{13}C-MRSI, the most developed hyperpolarized probe today is [1-^{13}C]-pyruvate, which enables the detection of activated lactate dehydrogenase in tumors (47). Isotopically labeled substrates and metabolites are clearly seen against naturally low abundance endogenous signals (e.g., 100%-enriched isotopomers versus 1.1% naturally abundant ^{13}C). Furthermore, hyperpolarization of ^{13}C substrates can be achieved by various techniques, including dynamic hyperpolarization (48) or parahydrogen induced polarization (49) and leads to a significant boost in the naturally low ^{13}C MRS signal. However, magnetization decays rapidly within minutes, necessitating fast ^{13}C MR imaging techniques. It has been shown that hyperpolarized ^{13}C-pyruvate/ lactate MRS(I) is superior to ^{18}FDG-PET (another metabolic imaging technique, see below) in detecting treatment response to novel targeted therapies and radiation (50, 51). Another approach to amplify MRS signals uses chemical exchange saturation transfer (CEST) MRI, which detects the exchange of protons from hydroxyl, amine, and amide groups to tissue water through the transfer of signal loss, with repeated proton exchange enhancing the effective signal in endogenous (52) and exogenous compounds (53) (Figure 1–D18). Amide proton transfer (APT) contrast, which is the CEST signal from endogenous cellular proteins and peptides, differentiates viable glioma from radiation necrosis (54). The use of *D*-glucose administration as a contrast agent for noninvasive CEST detection of tumors has been termed glucoCEST, and offers cancer detection with glucose as a biodegradable, nontoxic contrast agent (55). CEST measurements of regional pH, based on the clinically approved X-ray contrast agent iopamidol, have been applied in kidney and lung cancer models (56, 57). Another important nucleus for cancer characterization by MRS is ^{31}P for detection of phospholipid precursors, high energy phosphates and inorganic phosphate, which exhibits a pH-sensitive chemical shift in the physiological range (58), although it can be difficult to discriminate intra *vs.* extra cellular components. Meanwhile, ^{19}F-MR agents can offer superior chemical shift response (59). ^{19}F-MRI with perfluorocarbon agents has been used as an alternative to iron oxide T_2-MRI (see above and Figure 1–E22) to detect tumor-associated macrophages with the benefit of no endogenous background signal (60). Perfluorocarbons exhibit very high gas solubility and can serve as molecular amplifiers, as exploited to assess tumor pO_2 providing evidence for hypoxia, heterogeneity and differential regional response to interventions (28, 59, 61).

X-Ray Computed Tomography (microCT):

Micro-computed tomography (microCT) is a high-resolution 3-dimensional imaging technique; the physical principle of CT is based on scattering and absorption of x-rays by

tissues based on their electron density. There are essentially three levels of attenuation yielding color-coded contrast in CT: air (black), soft tissue (grey shades) and bones (white).

Anatomical microCT:

Compared to MRI, CT is inferior in distinguishing soft tissues/ organs, but the major strength of microCT lies in supreme high-resolution (<50 microns) fast imaging of lungs and bones revealing cancer lesions. Since bones are the most common metastatic site for major cancers (including breast and prostate), several studies reported the use of high-resolution (10 μm) microCT for detecting engrafted breast cancer cells in the bone (Figure 1–A4) (62). Inhibition of the development of osteolytic bone lesions by zolendronic acid has been reported in MDA-MB-231 breast xenograft mice, also identifying IL-1 as one of the key players for metastatic development (63–65). Due to the inherent contrast between air and tissue structures and the resulting attenuation of the x-rays passing through tissue, microCT is particularly well suited for providing high quality anatomical information in the lung. With the development of pre-cancerous lung conditions, including inflammation (66), fibrosis (67), and emphysema (68), and their progression to lung tumors (69–71), tissue structure becomes dense and can easily be differentiated from both normal lung and airspace. The use of 3-dimensional analysis to quantify tumor number, size and progression is advantageous over traditional histology (69) or macro-dissection of the lung to isolate tumors (70).

Vascular microCT:

Gated respiratory-holding techniques, fast acquisition times and the introduction of novel metal nanoparticles, such as ExiTron, allow lung microvasculature to be easily visualized, simultaneously with lung tumor detection (Figure 1–C13) (72, 73). The low radiation dose of modern instruments makes longitudinal microCT possible without long-term harm to animals (74). Recently, contrast-enhanced microCT has been applied to visualization and mapping of tumor vasculature in brain tumor and neuroblastoma mouse models (75–77).

Ultrasound (US) uses high-frequency sound waves and captures the ultrasound energy reflected from interfaces in the body (“echoes”) that separate tissue with different acoustic impedances, where the acoustic impedance is the product of physical density and velocity of sound in the tissue. Typically, a cyst appears sonolucent, because it gives few if any echoes (being mostly water), while liver and spleen have solid homogenous echo texture due to medium level echoes from the fibrous interstitial tissues. High-intensity echoes (increased echogenicity) are caused by calcification, fat and air interface; however, they do not propagate through bone. Among real time modalities, US features the highest frame rate up to 20,000 fps, enabling US-guided animal procedures, such as orthotopic cell tumor injections and left ventricular infusion of cancer cells to generate models of metastasis while avoiding lung engraftment (78, 79).

Anatomical US:

Pancreatic cancer is one of the most challenging mouse models for preclinical imaging. US provides fast precise quantification of pancreatic tumor burden longitudinally and without contrast administration (Figure 1–A6) (80, 81).

Vascular US:

US is also an excellent technique to assess tumor vasculature, e.g., Doppler US measures the speed and direction of flowing blood and has revealed vascular response to anti-angiogenic and Notch therapies in an orthotopic renal cell carcinoma mouse model (82) as well as irradiated rat fibrosarcoma tumors (83). Considerable attention has been given to the development of US-specific nanoparticles and microbubbles, which may be used both for vascular imaging and as theranostic drug carriers. The latest include VEGFR2 targeted microbubbles (84), oxygen microbubbles (85, 86) and US-destructible microbubbles for better delivery of paclitaxel-loaded nanoparticles in pancreatic cancer models (87). Acoustic Angiography (AA) is another contrast enhanced ultrasound technique, which uses the super-harmonic signals from microbubbles to produce high-resolution maps of vasculature with exceptional contrast since tissue yields no signal. Furthermore, AA can provide quantitative measurements of vascular density, blood perfusion, and vessel morphology, helpful to evaluate response to anti-angiogenic therapy in cancer (82). Quantitative US (QUS, (88)) is obtained from B-mode images and raw radiofrequency data and has been used to examine treatment response. Attenuation coefficients (ATN) and backscatter coefficients (BSC) can be derived (89). On the other hand, ultrasound elastography can visualize and quantify tissue stiffness noninvasively (90). These data can be used as a potential biomarker to assess changes in the tumor microenvironment, particularly changes affecting the extra cellular matrix (ECM), which may affect treatment efficacy (91, 92).

Photoacoustic imaging (PAI) represents the newest addition to the commercial armamentarium for pre-clinical imaging studies and progressively experimental investigations in man (93, 94). PAI exploits spectrally selective pulsed laser excitation of chromophores generating local thermoelastic tissue expansion, which is detected based on the resultant ultrasound acoustic waves, analogous to lightning generating thunder. Application of multiple wavelengths allows spectral discrimination, which has been applied to endogenous molecules such as oxy- and deoxyhemoglobin (HbO_2 and Hb) and melanin, and exogenous agents such as organic dyes, gold nanoparticles and genetically encoded proteins (95, 96). Indeed, spectral unmixing allows multiple materials to be detected simultaneously. The technology is particularly rapid, typically achieving single slice images in <100 ms, but usually images are acquired at multiple wavelengths, and signals may be averaged so that a typical acquisition time is 1–2 s. Gating may become relevant for assessing rapid changes in tissues subject to motion (97). Selection of an appropriate non-negative data reconstruction model is vital and choice of filters can enhance signal to noise (98, 99).

Various commercial instruments are optimized for in-vivo microscopic, mesoscopic, whole mouse tomographic and human applications, and may incorporate additional ultrasound excitation to enhance anatomical discrimination with typical spatial resolution approaching 100 μm at depths up to 5 cm.

The most effective application is assessment of tumor vasculature based on the ability to identify and quantify relative Hb and HbO_2 (Figure 1–B9) with effective studies of antiangiogenic therapy (100), acute vascular disruption induced by combretastatin (101,

102) and potentially prognostic observations following tumor irradiation (103). It appears that response to an oxygen breathing challenge characterized as ΔSO_2 is more closely related to perfusion and hypoxia than baseline static parameters (102), *e.g.*, low CAIX expression correlated with higher ΔSO_2^{MSOT}. Blood volume and perfusion may be effectively examined using contrast agents such as indocyanine green (ICG) (102, 104) or the liposomal formulation Genhance (105). Small molecule dyes may be incorporated in targeted liposomal formulations or used to directly label antibodies for detection of tumors or revealing receptor expression (106). Gold nanoparticles (which could also be used in microCT) exhibit exceptionally high photoacoustic activity based on surface plasmon resonance and may be tuned to wavelengths in the range 600–1000 nm based on size and shape (96, 107). Additional innovations include "smart" activatable probes, *e.g.*, sensitive to β-galactosidase activation (108) and genetically encoded proteins such as BphP1 (109). PAI essentially bridges two modalities to exploit spectrally selective optical excitation and robust spatial detection using ultrasound. It is very much an emerging technology.

Optical Imaging: Bioluminescence and Fluorescence (BLI and FLI):

Two decades after its invention, in-vivo optical imaging is now a well-established standard method to non-invasively monitor biological activity in mouse (and rat) research models. Optical imaging includes four molecular imaging modalities: BLI, FLI, chemiluminescence and Cherenkov imaging. The relatively low threshold of implementation, as well as the high sensitivity of in-vivo BLI, make this whole body, non-invasive imaging technique a go-to method in preclinical research (Figure 1–A5 & E19) (62, 110). Beyond tracking tumor growth and regression via constitutive firefly luciferase expression for drug efficacy determination, the toolbox for this molecular imaging technique has vastly expanded. Bioluminescent enzymes can be used to genetically tag cells, viruses, bacteria, gene therapy, and now also antibodies and their fragments (111). These enzymes such as firefly, renilla, gaussia and Nanoluc luciferases can be constitutively or inducibly expressed, and as such used for ratiometric imaging, gene expression studies, or dual labeling purposes (*e.g.* tracking T-cells infiltrating tumor) (12, 112–114) (Figure 1–E19). Split luciferases to evaluate protein-protein interaction, as well as split luciferin substrates to monitor apoptosis have been designed and are utilized to evaluate mechanism of action (115). Potential drawbacks of BLI are the need for cell transfection and delivery of reactive substrate. Luciferin effectively crosses barriers such as blood brain and placenta and its very delivery to tissue has been used to assess selective vascular destruction in tumors (101, 116). Bioluminescent resonance energy transfer (BRET) constructs such as Antares, which red shifts the lower wavelength Nanoluc luciferase for better in-vivo sensitivity, are also available (117). Chemiluminescent compounds, substrates and sensors are luminophores that emit red shifted light upon chemiexcitation have been reported for detection of H_2O_2, H_2S, formaldehyde, beta-galactosidase and nitroreductase activity (118–121). Dr. Cherenkov received the Nobel Prize in 1958 for his discovery of the bluish hue of light emitted by decaying radioisotopes. This same light emission can be detected by screening mice injected with diagnostic radioisotopes such as ^{18}FDG in an in-vivo optical imaging system, adopting the epithet of a poor man's PET scanner (122) and may also be relevant for radiation dosimetry (123). FLI on the other hand features both genetically encoded fluorescent

proteins (FPs) and fluorescent dyes. The powerful combination of BLI and FLI is exemplified by Zeng *et al.* (124) (Figure 1–F23), illustrating the tracking of fluorescent micelles to bioluminescent brain tumors. In comparison with BLI however, the contrast to noise is less with fluorescence due to non-specific autofluorescent noise originating from innate proteins in tissue. This issue is being combatted with the discovery of red-shifted FP's for better in-vivo sensitivity, an initiative led by Nobel laureate Dr. Roger Tsien (125). A second window of opportunity for in-vivo FLI is currently being explored in the short wavelength infrared (SWIR) using ultra-bright near-infrared-IIb rare-earth nanoparticles. Here, tissue absorption and light scattering are significantly reduced (126) rendering higher resolution, higher depth penetration images. Crafty alternatives have also been invented in which fluorescent sensors are quenched until activated by an enzymatic reaction (*e.g.*, cathepsin, matrix metalloprotease, neutrophil elastase, etc.) or in which fluorophores shift wavelength upon binding their target (127). A great advantage of fluorophores is that they are also readily detectable ex-vivo for histopathological evaluation. This is highly translational, and intrasurgical fluorescence imaging is actively being explored to both highlight tumor burden, and also improve tumor margin of resection (128). Preclinical optical cancer imaging begs for anatomical context, prompting co-registration with anatomical imaging modalities such as X-ray, microCT, MRI or the recently developed robotic ultrasound, which features inexpensive, exogenous contrast free 3D soft tissue resolution (78).

PET and SPECT:

Nuclear medicine images are produced by giving the animal short-lived radioactive isotopes and detecting their decay using a gamma camera (SPECT) or positron emission (PET) scanner, revealing spatial and temporal distribution of target-specific radiotracers and pharmaceuticals. An extensive array of radiopharmaceuticals, or molecular probes exist (based on ^{11}C, ^{13}N, ^{15}O, ^{18}F, ^{124}I, ^{64}Cu, ^{68}Ga, ^{89}Zr for PET and ^{123}I, ^{99m}Tc, ^{201}Ti, ^{111}In for SPECT) to image diverse aspects of tumor physiology and biology. Data can reveal properties such as glucose metabolism, blood volume and flow, tissue uptake, receptor binding, and oxygen utilization. Since both modalities have relatively low spatial resolution, CT is usually added for an anatomical overlay of the biodistribution of the radio-labeled probe.

Metabolic PET:

^{18}FDG-PET is the most established metabolic cancer imaging approach both pre-clinically and clinically. Most tumors have a highly glycolytic phenotype (the Warburg effect) providing the basis for increased uptake and accumulation of the radioactive glucose analogue ^{18}FDG, as shown in various mouse models of leukemia, pancreatic, lung, colorectal, breast, prostate cancers (Figure 1–D15) (51, 129–132). Other tracers have recently been introduced to elucidate abnormal metabolic phenotypes, including, either ^{11}C- or ^{18}F-, acetate (mitochondrial metabolism) (133), choline (membrane phospholipids) (133, 134), amino acids in brain tumors (glutamine, tyrosine or methionine, Figure 1–D16) (135–138).

Physiological PET:

Several essential ^{18}F-labeled tracers should be mentioned here as potential (although not entirely specific) markers for tumor cell proliferation (^{18}F-fluorothymidine, ^{18}FLT) and hypoxia (18F-fluoroazomycin arabinoside, ^{18}F-FAZA, and ^{18}F-fluoromisonidazole, ^{18}F-MISO). Radioactive thymidine is readily incorporated into DNA synthesis, making an increased uptake of ^{18}FLT visible on animal PET and correlating with increased ADC on diffusion weighted MRI, albeit exhibiting low specificity (139–142). ^{18}F-MISO is trapped in hypoxic areas as compared with BOLD and TOLD MRI (Figure 1–B10) (143). While ^{18}F-MISO has been tested for many years, its uptake selectivity is suboptimal and many other potential hypoxia imaging agents are under development and evaluation (e.g., ^{18}F-FAZA shows more rapid background clearance (144, 145).

Cellular PET:

With the development of check-point inhibitor and immunotherapies, significant efforts have been dedicated to develop so-called “immunoPET”. Several T-lymphocyte targeting molecules were radiochemically labelled with long-lived radionuclides (such as ^{64}Cu, ^{68}Ga, ^{89}Zr). Following intravenous injection intra-tumoral accumulation of T-lymphocytes has been non-invasively detected in response to check-point inhibitor treatment (Figure 1–E20) (146–148).

Molecular PET/SPECT:

Specific molecular targets have been visualized using PET- or SPECT-based peptides, antibodies and receptor-binding ligands. One of the most explored is hormone imaging, ^{18}F-fluoroestradiol (^{18}FES) PET, as used for pre-clinical and clinical imaging of ER+ breast and ovarian cancer (Figure 1–F24) (149–152). Recent examples of hormone imaging include PET of androgen receptor in rat brain (153). Several ^{111}In/^{203}Pb labelled peptides for SPECT (154) and ^{68}Ga-MSH for PET (155) have been developed to target the melanocortin-1 receptor in melanoma mouse models (Figure 1–F25). A ^{203}Pb/^{212}Pb theranostic pair has been reported for PSMA-based α-particle targeted radiopharmaceutical therapy in advanced prostate cancer (156).

Other notable imaging technologies include MPI and ESR. ***Magnetic Particle Imaging (MPI)*** is an emerging imaging modality that involves iron oxide nanoparticles. Unlike MRI, MPI measures electronic moment of particles, which is more sensitive than measuring changes in proton relaxation by MRI. The detection is linear, sensitive (ng of iron per voxel) and has a high signal to background ratio. Using MPI of iron oxide particles, kinetics of accumulation of nanoparticles in rat tumors (157) and kinetics of drug release in mouse breast tumors (158) were studied. Further applications of MPI are dependent on improving the acquisition speed and resolution, as well as improving circulation and targeting properties of nanoparticles.

Electron paramagnetic resonance (EPR), also termed Electron spin resonance (ESR) has been a research tool for many years, but remains somewhat esoteric in cancer research, largely due to lack of available instrumentation. It directly detects free radicals but the extremely high frequencies tend to limit tissue penetration, though effective studies have

been performed in mice and human teeth and tattoos (159). The most popular application has been based on imaging signal line width and relaxation mechanisms, which may be directly responsive to the presence of oxygen and hence pO_2. Reporter agents may be injected directly into tumors (e.g., India ink or chars (160), or infused systemically (OX63-oxygen-measuring spin probe, coincidently the same material used to achieve hyperpolarization of ^{13}C substrates for NMR) (161). Sensitivity to oxygen can be particularly high at very low, radiobiologically relevant pO_2 values (0–15 Torr) and significant correlations have been observed between pO_2 values and radiation response (50, 160–162). A significant drawback of EPR is the lack of integrated anatomical information, generally requiring that separate MRI be performed and co-registered.

Image-Guided Irradiation:

Radiation plays an important role in cancer therapy; radiation-based therapy has been applied to animal models for decades and recently has undergone significant improvement in terms of applying multi-modality imaging to guide radiation planning (163, 164). Radiation kills cancer cells by damaging DNA, either directly or indirectly through the creation of reactive oxygen species. Because radiation kills both cancer cells and healthy cells alike, various methods are used to increase the tumoricidal effects of radiation while minimizing damage to the surrounding normal tissue, including spatial modulation of the dose distribution to conform to a specific target region. While such conformal dose distributions allow for significant reductions in normal tissue toxicity, they also require onboard image guidance systems to ensure the tumor is in the correct location when the radiation beam is turned on. Modern animal irradiators incorporate multi-modal imaging detectors to precisely guide the radiation, combining the ability to deliver targeted radiation treatments using a 225 kVp, gantry-mounted x-ray tube with digital radiography, fluoroscopy, cone-beam computed tomography (CBCT), and bioluminescence imaging (BLI) (164, 165). Image-guided irradiation has been successfully applied even for small orthotopic head-and-neck and lung lesions in tumor-bearing mice (166, 167). The software also allows import of existing imaging data sets from other modalities such as MRI – which often plays a crucial role for irradiating intracranial brain tumor models (9, 163)

Image Analysis and Quantitative Biomarkers:

There is increasing interest in using imaging to develop non-invasive quantitative imaging biomarkers (surrogate endpoints) for cancer characterization. Indeed, most imaging read-outs are provided in both qualitative and quantitative form (Table 1B) (168). This is especially true for MRI, CT and ultrasound, due to their high spatial resolution to provide precise tumor dimensions as well as number of suspicious lesions/ metastases (169, 170). The well-established mathematical modeling algorithms for tracer kinetics allow quantification of tumor vasculature based on gadolinium, nanoparticle and microbubble uptake for MRI, CT and US, respectively (32, 34). The biomarkers include the exchange rate constants (K^{trans}), which reflect the efflux rate of gadolinium contrast from blood plasma into the tissue/ tumor extravascular extracellular space (EES), the volume of contrast agent distribution V_e, or simply the area under enhancement curves after the administration of contrast (19, 171–174). Finally, physiological MRI provides established quantitative end-

points in the form of apparent diffusion coefficients (ADC) from diffusion-weighted MRI: low ADC ($0.5–0.8\times10^{-3}$ mm^2/sec) indicates densely cellular aggressive tumors, while treatment-induced necrosis results in increased ADC up to 1.2×10^{-3} mm^2/sec, and radiation-induced edema's ADC as high as 2.2 (17, 19). PET and SPECT tracer uptake is usually reported as standardized uptake values (SUVs) which includes normalization to injected dose and accounts for radionuclide decay (129, 130, 175). Several studies report ratios of signal intensities of the tumor-to-normal tissue (most often for brain tumors as tumor-to-brain ratios, TBR) (138, 174). Optical imaging (BLI and FLI) is rather semi-quantitative, but can provide signal intensities related to tumor volume or tissue perfusion (SIs) (11, 114), e.g., the change in light emission from a luciferase expressing tumors following an acute intervention such as a vascular disrupting agent provides an indication of vascular shutdown (101, 176, 177). Multimodality imaging ideally combines the advantages of each modality, while mitigating their deficiencies. Image registration is necessary when more than one imaging modality is used. Histology can often serve as the ground-truth for the validation of image-based biomarkers or new imaging modalities.

Identifying non-invasive biomarkers to be used clinically as surrogate endpoints is not only valuable, but also promising. The advent of machine learning and artificial intelligence in medical imaging has led to the field of radiomics (170, 178–181). Like genomics and other "-omics", radiomics allows quantifiable characterization of image features that provide a means to identify image-based biomarker surrogates for response to cancer treatment. Cameron *et al.* report a radiomics method, MAPS, based on Morphology, Asymmetry, Physiology, and Size (MAPS) using multi-parametric MRI (182). Most radiomics data have been reported for multicenter human studies, since a large number of subjects needs to be enrolled – the number of experimental animals in a single imaging study often being a limiting factor. As quantitative imaging and radiomics lead to more image-based biomarkers, standardization and assessment of reproducibility are becoming important and will require a centralized image archive for multi-center preclinical studies.

Future Directions in Translational Imaging:

Imaging is highly translational by nature and murine models have contributed enormously to the development of oncologic imaging methodologies (183). However, the complex, heterogeneous tumor microenvironment observed in human cancer is challenging to model in an immunodeficient animal system, particularly in terms of immunotherapeutic strategies. Lack of optimal pre-clinical models for testing is likely responsible for the dismal success rate (5–8%) of cancer therapeutics developed in murine models to eventually obtain FDA approval for use in human patients (184). Dogs with naturally occurring cancers provide an alternative, complementary system for preclinical cancer research. The recent completion of the sequencing of the dog genome has shown that most of their 19,000 genes are orthologous or similar to humans (185). Companion animals live in our homes and are exposed to similar environmental and lifestyle influences. Their cancers grow slowly in an immunocompetent milieu, allowing for complex carcinogenesis, genomic instability and immune avoidance to develop. Their size is such that serial biologic sampling can be performed before, during and after therapy. These patients are imaged in human equipment, allowing for standardization of imaging protocols, improved spatial resolution for more

accurate quantitative analysis and adequate quality assurance of biodistribution for novel imaging probes. Power Doppler ultrasound and contrast-enhanced ultrasound were used to demonstrate tumor vascular response to anti-vascular therapy in canine cancer patients non-invasive (186). There are several success stories to report today: ^{18}FDG- and ^{18}F-NaF PET/CT have been successfully used in canine cancer patients to detect head-and-neck cancer and bone involvement of the nasal cavity (Figure 1–F23) (187). An iodinated nanoparticle CT tracer initially developed and validated in a murine lung cancer model (described above (73)), has been successfully used in a CT study of companion dogs with spontaneous tumors (188). An anatomic and functional imaging probe for a novel immunotherapeutic was developed in dogs with spontaneous lymphoma (189). A recombinant oncolytic vesicular stomatitis virus that expresses a surface sodium-iodide symporter (NIS) protein and IFNβ was characterized. Based on clinical response to VSV-IFNβ-NIS therapy in dogs with T cell lymphoma, a phase I clinical trial in people has been started (NCT03017820) (189). In a follow up study, dogs administered VSV-IFNβ-NIS were evaluated to determine whether ^{18}F-tetrafluoroborate radiopharmaceutical that binds to the cell surface NIS can be used to confirm successful viral gene replication (190). Veterinary patients with naturally occurring cancers may assist in the development of new molecular imaging probes, shorten the approval process of oncologic therapies and create a mutually beneficial bridge between the fields of veterinary and human oncology.

In summary, multi-modal oncologic imaging has become a cutting-edge necessity in pre-clinical (animal) cancer research. Understanding the physical principles of each modality is essential for applying the correct non-invasive imaging protocol to an animal-based study. Development of imaging probes for multimodal imaging technologies is also an important scientific and clinical goal. Each imaging modality brings specific insights into oncological questions and allows researchers to follow the biology dictating the choice of the optimal reporter and imaging modality to best characterize cancer phenotype (191). The future also holds a big promise for PET/MRI (similarly to existing PET/CT) combining two powerful molecular, physiological and structural techniques into one scanner. Finally, we anticipate the introduction of novel predictive models and deep learning algorithms (192) in the near future for managing sophisticated and complex image data sets in animal models of cancer.

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Abbreviations:

ADC	apparent diffusion coefficient
AUC	area under the curve
BLI	bioluminescence imaging
BOLD	blood oxygen level dependent

CT	computed tomography
CE-CT	contrast enhanced computed tomography
DCE	dynamic contrast enhanced
EPR	electron paramagnetic resonance
ESR	electron spin resonance
FLI	fluorescence imaging
fMRI	functional MRI
FOV	field of view
HbO_2	oxy-hemoglobin
K^{trans}	volume transfer constant
MPI	magnetic particle imaging
MRA	magnetic resonance angiography
MRI	magnetic resonance imaging
MRS	magnetic resonance spectroscopy
MSOT	multispectral optoacoustic tomography
NP	nanoparticle
PAI	photoacoustic imaging
PDX	patient-derived xenograft
PET	positron emission tomography
ΔR_1	change in longitudinal relaxation rate ($R_1=1/T_1$)
ΔR_2*	change in apparent transverse relaxation rate ($R_2=1/T_2$)
SI	signal intensity
SO_2^{MSOT}	hemoglobin oxygen saturation measured using MSOT
SPECT	single photon emission computed tomography
SUV	standard uptake value
tBV	tumor blood volume
TOLD	tissue oxygen level dependent
US	ultrasound
V_e	extra cellular- extra vascular space

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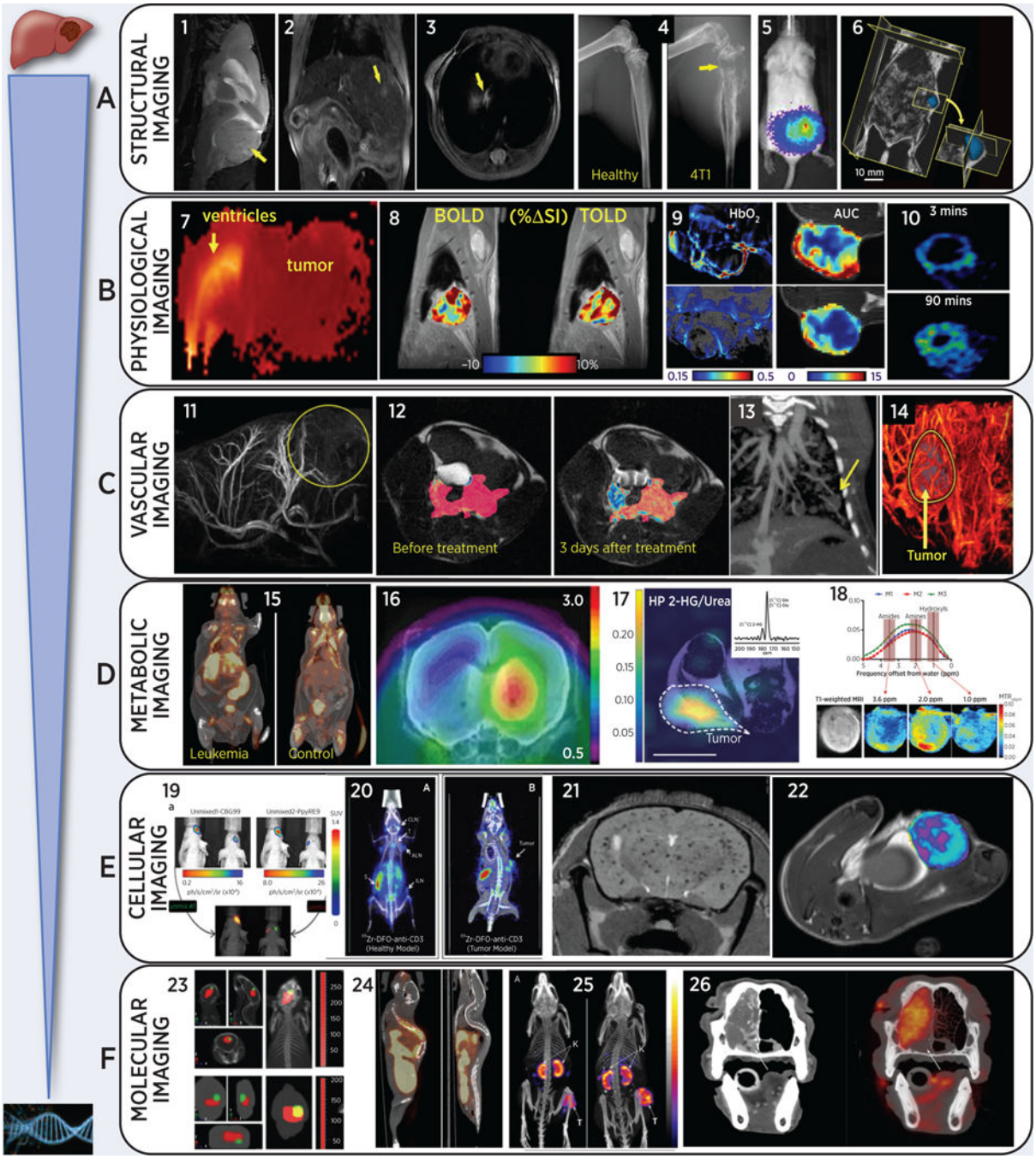

Figure 1:

Representative multi-modality images of animal cancer models (from left to right): (A) Anatomical cancer detection in mouse models: (1) T_2-weighted MRI of pediatric cerebellar brain tumor (medulloblastoma) pdx; (2) gadolinium-enhanced T_1-MRI of an orthotopic liver HCC; (3) proton-density MRI of a lung metastasis from breast cancer; (4) microCT of bone metastasis from engrafted breast cancer cells, adapted from (62); (5) BLI of multi-organ breast cancer metastases, adapted from (62); (6) 3D-ultrasound of pancreatic cancer in a genetically modified LSL-Kras mouse, adapted from (80).

(B) Physiology-based images in rodent cancer models: (7) high ADC (brain edema and ventricle hydrocephalus) and low ADC (highly proliferative medulloblastoma mouse pdx) from diffusion-weighted MRI; (8) blood and tissue oxygen level dependent (BOLD and TOLD) MRI in response to O_2 gas breathing challenge in orthotopic human A549 lung tumor xenograft in nude rat, adapted from (23); (9) left: photoacoustic imaging of subcutaneous A549 human lung tumor growing in leg of nude rat showing endogenous HbO_2 concentration before (upper) and 48 hrs after (lower) administration of vascular disrupting agent (VDA), based on multiple wavelengths (MSOT), while breathing O_2 and right: corresponding DCE MRI showing area under the curve reflecting reduced perfusion after VDA (10) ^{18}F-MISO (hypoxia tracer) PET in a syngeneic Dunning R3327-AT1 rat prostate tumor, adapted from (143)

(C) Imaging tumor vasculature in-vivo: (11) high-resolution magnetic resonance angiography (MRA) after gadolinium injection in an orthotopic rat isograft C6 glioma model; (12) DCE-MRI during gadolinium injection in mouse TRAMP model for prostate cancer, adapted from (33); (13) contrast-enhanced microCT of lung vasculature and small lung tumor using liposomal-iodinated contrast agent, adapted from (72); (14) US enhanced with microbubbles reveals high perfusion in the rim of a flank pancreatic cancer xenograft in a mouse.

(D) Imaging tumor metabolism non-invasively: (15) abnormal ^{18}F-FDG uptake in spleen, liver, and lymph nodes in transgenic leukemic (left) *vs.* control mouse, adapted from (129); (16) increased GBM uptake of ^{18}F-ethyltyrosine (^{18}F-FET) without (left) and with bevacizumab treatment in an orthotopic U87 glioma mouse model, adapted from (138); (17) Representative heatmap of spectral data from a mouse with a mutant IDH1 tumor xenograft following injection of hyperpolarized [1-^{13}C]-glutamine showing accumulation of 2-HG in the tumor region only, which was referenced and normalized to a 5 mM [1-^{13}C] urea phantom. Dotted lines highlight the tumor, and the white line at the bottom represents 10 mm for scaling, adapted from (46); (18) In-vivo CEST-MRI of MDA-MB-231 breast tumor xenografts showing representative CEST MRI maps (top row, A), T1-weighted RARE MRI (bottom left, B), and MTR_{asym} for three individual mice with orthotopic human MDA-MB-231 breast tumor xenografts, which were labeled M1 for mouse 1, M2 for mouse 2, and M3 for mouse 3. CEST shifts of amide, amine, and hydroxyl resonances are highlighted in C, adapted from (52);

(E) Cellular tracking using non-invasive imaging in mouse cancer models: (19) Dual reporter bioluminescence imaging using spectral unmixing algorithm. CBG99 cells were transplanted into the right striatum, PpyRE9 cells into the left striatum of the nude mouse on the right. A spectral unmixing algorithm was applied in order to select green light from CBG99 and red light from PpyRE9, adapted from (191); (20) immune-PET to image T-lymphocytes using ^{89}Zr-anti-CD3 in normal and BBN975 bladder cancer tumor-bearing mice, adapted from (147); (21) T_2-weighted brain MRI of ferumoxytol-labeled breast cancer cells after intra-cardiac injection, adapted from (35); (22) T_2-weighted maps for macrophage imaging after ferumoxytol injection in inflamed mammary gland tumor mouse model, adapted from (37);

(F) Molecular imaging of tumor-specific molecules: (23) tracking fluorescent micelles (red signal) to bioluminescent brain tumors (green) in anatomical context (124); (24) whole-body ^{18}F-estradiol (FES) PET/CT of estrogen receptor in ER positive and negative bone

metastases in mouse models of breast cancer; (25) whole body SPECT/CT with ^{111}In-MSH peptide (melanocyte stimulating hormone) to image melanocortin-1 receptor in mouse B16/F1 melanoma model, adapted from (154); (26) CT (left) and ^{18}FDG-PET of nasal adenocarcinoma in a canine cancer patient (a 10-year old standard poodle), adapted from (187).

Table 1:

(A) physical principles of the main pre-clinical imaging modalities and their basic characteristics; (B) the ultimate guide for choosing a specific imaging platform in a cancer research study design.

Table 1A Modality	**Physical Principles**	**Whole Body/ Target Organ**	**Resolution Scale**
MRI/ MRS	External magnetic field; nuclear spin; radio wave pulses (for magnetization of hydrogens in tissue water/ metabolites)	4 – 6 cm FOV: brain, heart, liver, pancreas, muscle	35 – 150 microns
microCT	3-Dimensional X-ray beam absorption and scattering	Whole body/ lung, bone	10 – 50 microns
Ultrasound (US)	Reflection of high-frequency sound waves	2–4 cm FOV: heart, pelvic, liver, pancreas, OBGYN	60 – 120 microns
Photoacoustic (PAI, MSOT)	Spectrally selective near infra-red light excitation of chromophores inducing sound waves providing tomographic images; notably oxy-deoxyhemoglobin, exogenous 800CW tagged agents and gold nanoparticles	Tomographic slices of whole mouse or larger animal to 4 cm depth; breast, thyroid	150 microns; 100 ms
Optical: BLI and FLI	Light emitting chemical reaction, often enzyme facilitated, e.g., luciferin/luciferase; photo stimulated fluorescent chromophores	Whole body	mm- depth dependent
PET/SPECT	Decay of short-lived radioactive beta+ and photon emitters	Whole body	1.0 – 1.8 mm

Table 1B: Tumor Etiology	**Appropriate Imaging Modality to Assess Tumor Characteristics**	**Quantitative Imaging Biomarkers**
Dimensions	CT, T1/T2-MRI, Ultrasound	Tumor volume, mm^3 Tumor diameter, mm
Cellularity Proliferation	Diffusion-weighted MRI ^{18}FLT-PET	Apparent diffusion coefficient (ADC) Standard uptake values (SUV)
Metastases	CT, MRI ⇨ BLI, PET	Number of lesions ⇨ qualitative
Vascularity/ Oxygenation/ Hypoxia	MRA, DCE, CE-CT, PAI sO_2-MSOT oxygen-enhanced MRI (BOLD/TOLD), ^{18}F-MISO, ^{18}F-FAZA PET	Exchange rate constants K^{trans}, V_e ΔR_2* maps, ΔR_1, AUC, tBV HbO_2; SO_2^{MSOT}; SUV
Metabolism/Tumor pH	PET, FLI ^{1}H-MRSI, hyperpolarized ^{13}C-MRSI, ^{31}P-MRS, ^{19}F-MRS pH: ^{31}P-MRS, CEST-MRI	SUVs, Signal intensities (SIs) Metabolite concentrations, metabolite ratios, metabolite maps Intra-extracellular pH values and pH maps
Inflammation Redox Imaging	immunoPET, Iron Oxide NP T_2-MRI, PFC ^{19}F-MRI, EPR	SUVs, ΔT_2 relaxation times SIs
Cellular Tracking	BLI, ^{19}F-MRI, Iron Oxide T_2-MRI, PET	SIs, SUVs
Molecular Targets	SPECT, PET, BLI and FLI imaging	SUVs, SIs ⇨ qualitative